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NATURE AND ROLE OF PROTEINACEOUS HORMONAL FACTORS ACTING DURING PUPARIUM FORMATION IN FLIES

P. SIVASUBRAMANIAN,[1] S. FRIEDMAN AND G. FRAENKEL

Department of Entomology, University of Illinois, Urbana, Illinois 61801

In 1969, Zdarek and Fraenkel found that hemolymph from early puparia or neurosecretory material from the pars intercerebralis of the brain would greatly accelerate puparium formation in flies. After further investigation of the puparium-accelerating reactions from the CNS and the hemolymph, Fraenkel, Zdarek and Sivasubramanian (1972) proposed two alternative models for the neuroendocrine events that occur during pupariation in flies. The first implied that ecdysone stimulates the synthesis and/or storage and release into the hemolymph of two proteinaceous factors, one leading to retraction of the anterior segments into the body (X_r), and the second causing tanning (X_t). A different neurosecretory product from the central nervous system (CNS) accelerates the release of these proteinaceous factors. The second model was based on an assumption of the identity of the acceleration factors in the CNS and hemolymph. According to this a neurosecetory product from the brain is stored in peripheral nerves or at nerve endings before the critical period, and ultimately released into the hemolymph as the X-factors by the action of ecdysone. Injections of brain extracts supply the X-factors into the blood sooner and thus accelerate pupariation.

The schemes suggested by Fraenkel *et al.* (1972) raised several questions. Are there really two distinct protein factors controlling retraction of the anterior segments and tanning? How are the hemolymph factors related to the brain factors? Do they originate from the brain or are they synthesized in some other tissue(s)? If the X-factors do exist, to what extent can they replace ecdysone during pupariation? The subject of this investigation is to seek answers to these questions and to clarify the existence, nature and action of the X-factors during pupariation in the larva of the fleshfly, *Sarcophaga bullata* Parker. A fuller presentation of this work is given elsewhere (Sivasubramanian, 1973).

In the present study X_r (the retraction factor) and X_t (the tanning factor) will be referred to, respectively, as ARF (anterior (segments) retraction factor) and PTF (puparium tanning factor). Since the bioassay for the factors depended on the acceleration of the respective processes they were originally called acceleration factors by Fraenkel *et al.* (1972). However, all available evidence points to the fact that retraction and tanning cannot occur in the absence of these factors. Hence they will be referred to in this paper as hormonal factors instead of acceleration factors.

Materials and Methods

Experimental animal

The insect used throughout this study was *Sarcophaga bullata.* Staging the larvae for use as hosts and donors in bioassay: *Hosts,* about 3–4 hours before pu-

[1] Present address: The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138.

pariation of the larva of *Sarcophaga bullata* the cuticle between and around the posterior spiracles begins to tan. This is a clear indication of forthcoming pupariation and a good time to obtain larvae of uniform age. Larvae of this stage, called red-spiracle larvae, were found to be most responsive as hosts for experiments on pupariation (Zdarek and Fraenkel, 1969) and, unless otherwise stated, have been used as hosts in this study. *Donors*, according to Fraenkel *et al.* (1972), the hemolymph from the orange puparium of *Sarcophaga bullata* (about 1 hour after the formation of the white puparium) is highly active with respect to the X-factors that are involved in immobilization of larvae (during puparium formation) by retraction of anterior segments and tanning. Therefore, orange puparia were used as donors of hemolymph. In the same report it was shown that the pupariation-accelerating activity of the CNS changes very little from the mature larva (crop-emptied, post-feeding stage about 10–12 hours before pupariation) until 16 hours after puparium formation. Therefore, mature larvae were used as CNS donors.

Method of injection

Host larvae were immobilized on ice and injected according to the techniques of Zdarek and Fraenkel (1969), using glass pipettes with a tip diameter of about 40 μ.

Ligation techniques

Ligatures with heavy duty cotton thread were applied well behind the brain-ring gland complex (approximately on the 6th segment) in such a way that the neural and endocrine sources were isolated anterior to the ligation.

Bioassay methods

Intact and ligated host larvae were used in the bioassays for ARF and PTF. The activities of both factors were determined in intact larvae but only tanning could be scored in ligated larvae.

Scoring the results

After injection the larvae were left on filter paper in Petri dishes, and examined every 10 minutes. In intact larvae the time taken for the retraction of the anterior segments and that taken for tanning were expressed as percentages of controls injected with *Drosophila* Ringer solution. In the ligated red spiracle larva there is already enough ecdysone to induce tanning on both sides of the ligature, but the anterior part always tans before the posterior part because the CNS in the former liberates certain pupariation acceleration factors (Zdarek and Fraenkel, 1969). Thus, in the Ringer-injected controls, the quotient of the pretanning period in minutes of the posterior part over the anterior part (P/A) is always more than 1. If the hind parts are injected with material containing the hormonal factor they tan before the anterior part and the quotient of P/A is less than one.

In order to express activities quantitatively, increasing amounts of protein with ARF and PTF activities were injected into intact and ligated hind parts of red-spiracle larvae, respectively. The time taken for retraction of the anterior segments under the influence of ARF, expressed in terms of a percentage of control

time, was plotted as a function of the amount of protein injected. Similarly, the time taken for tanning after PTF injection, as expressed by the P/A ratio of the ligated larva was plotted as a function of the quantity of protein injected. Each curve was linear over a portion of its range, so the quantity of protein necessary to effect either retraction of anterior segments or tanning at the mid point of the linear part of the curve was considered as 1 unit of activity. This was at 50% of control time for ARF and at a P/A ratio of 1 for PTF.

Protein was determined according to the method of Lowry, Rosebrough, Farr and Randall (1951), using crystalline bovine serum albumin (BSA) as a standard.

Isolation of active factors

All procedures involved in the fractionation of hemolymph and CNS were carried out at 2° to 4° C. In the collection of hemolymph and CNS, the hemolymph was expressed from cut anterior ends of orange puparia into a centrifuge tube containing a few crystals of phenylthiourea. In order to dissect the CNS, mature larvae were immobilized on ice and their mouth hooks plus the first three body segments chopped off. A gentle pressure on the body brought out the CNS with a little bit of fat body. This was teased away in Ringer solution and the CNS were preserved in acetone and stored at 0–4° C until use. Then the acetone was decanted and the dry CNS's were homogenized in Ringer solution at a concentration of 100 CNS per ml and centrifuged in the cold (5° C) at 105,000 *g* for 50 minutes in a Beckman Model L-2 ultracentrifuge. The supernatant (cytosol) was used for further fractionation.

Enzyme hydrolysis. Approximately 10 mg of "Enzite-Try" (Immobilized trypsin, Miles-Seravac (PTY) Ltd, Maidenhead, Berks, England) was added to 1 ml of the sample and the mixture incubated at 37° C for 1–2 hours. After incubation the enzyme was sedimented by centrifugation at 2000 *g* for 10 minutes and the supernatant tested for activity. The sample was incubated with an equal volume of 0.1% pepsin in 0.01 N HCl for 2 hours at 37° C. Then the enzyme was inactivated by heating to 100° C for 3 minutes.

Ultrafiltration. Ultrafiltration of soluble fractions was performed with Amicon permselective membranes (Amicon Corp., Lexington, Massachusetts 02173) graded in terms of molecular weight cut off levels.

Gel filtration. Bio-Gel P-300 (100–150 mesh) (Bio-Rad Labs., Richmond, California 94804) was hydrated with Ringer solution and packed in a column of 1.6 × 36 cm size using the method suggested by the manufacturers. About 2 ml of sample, consisting of a total of about 250 mg of protein, was applied on the column and eluted with Ringer solution. One-ml fractions were collected at a rate of 3 ml per hour and tested for activity as previously described. Bovine liver catalase (Sigma), beef heart LDH (Sigma) and *Escherichia coli* alkaline phosphatase (Worthington) were used as markers for molecular weight determinations.

Gel electrophoresis. Seven per cent (W/V) polyacrylamide gels were prepared in gel tubes 9 × 0.7 cm, and disc electrophoresis carried out using the techniques outlined by Davis (1964). Current during the run was 3 mAmp per tube. Active fractions of hemolymph (from Bio-Gel P-300 column) or CNS (cytosol fraction retained by XM-300 filter) equivalent to about 500 μg protein were used. After electrophoresis gels were fixed for 30 minutes in 12.5% trichloroacetic acid and

stained with Coomassie brilliant blue solution (one volume of 1% aqueous CBB solution plus 19 volumes of 12.5% TCA). Gels were destained in 10% TCA and stored in 7.5% acetic acid.

For preparative electrophoresis the method was exactly the same as that described for analytical electrophoresis except that at the end of a run the gels were neither fixed nor stained. Instead they were sliced and the proteins extracted with Ringer solution overnight in the cold. After concentration by filtering through the Amicon membranes the supernatants were used for assay.

The technique of electroelution used was that of Maizel (1964) with certain chamber modifications. Electrophoresis through 7% polyacrylamide was employed to separate proteins, but was continued until each protein had completely traversed the gel, and moved out into a small elution chamber at the bottom of the gel tube. Buffer was continuously pumped through the chamber and the fractions were collected separately using a fraction collector.

Estimation of molecular weights. The molecular weights of pure active samples were determined by SDS (sodium dodecyl sulfate)-gel electrophoresis using a modification of the method of Weber and Osborn (1969).

Five volumes of the sample or a standard (yeast alcohol dehydrogenase, bovine liver catalase and bovine serum albumin, all from Sigma Chemical Corp. St. Louis, Missouri, and proteins of potato yellow dwarf virus with known molecular weights obtained from Dr. R. McLeod, Department of Botany, University of Illinois, Urbana), were mixed with 1 volume of 0.05 M sodium phosphate buffer containing 10% SDS, 10% mercaptoethanol and 1 mg of urea and boiled for 5 minutes. They were then prepared for the run as follows: Protein solution after boiling, 10 μl (about 10 μg); 0.01 M phosphate buffer containing 1% SDS and 1% mercaptoethanol, 25 μl; 0.05% bromophenol blue in water, 5 μl; Mercaptoethanol, 5 μl; 40% sucrose in water, 2 drops. The above solutions were mixed and run on a 7 × 0.6 cm column of 7% acrylamide containing 0.1% SDS and 8 M urea at 6 mAmp per tube at room temperature for about 4 hours. The gels were stained for 2 hours in 0.25% Coomassie brilliant blue in methanol, water and glacial acetic acid (5:5:1). After staining the gels were kept for at least 30 minutes in a destaining solution consisting of glacial acetic acid, methanol and water (3:2:35) and then destained electrophoretically for 2 hours in the same solution.

Results

In all the following experiments the volume of sample injected per host was 8 μl. The desired amounts of proteins to be injected were made up to 8 μl with Ringer solution and then injected.

Unfractionated sample

Hemolymph from orange puparia was collected and centrifuged in the cold for 10 minutes at 700 *g*. Injection of the supernatant plasma into intact red-spiracle larvae indicated that at least 105 μg of hemolymph protein are required to effect retraction of anterior segments in 50% of control time (1 ARF unit). About 3 times more protein had to be injected to observe PTF activity in ligated larvae (Table 1).

TABLE I

Effect of injection of various quantities of hemolymph from orange puparia into intact and ligated hind parts of red-spiracle larvae of S. bullata. *(One ml of hemolymph contains about 205 mg of protein.) The figures are means from 20 observations*

Quantity of hemolymph protein injected (μg)	Retraction % Control*	Tanning	
		% Control**	P/A***
0 (Ringer sol.)	100	100	1.33 ± 0.03
820	24.5 ± 0.74	55.2 ± 2.3	0.73 ± 0.034
410	23.7 ± 0.86	64.8 ± 5.1	0.79 ± 0.036
300	25.6 ± 0.67	65.9 ± 2.8	1.05 ± 0.006
205	27.0 ± 0.93	69.4 ± 5.8	1.18 ± 0.047
105	52.6 ± 1.4	76.6 ± 3.6	1.29 ± 0.02
80	75.1 ± 2.3	89.0 ± 4.2	1.26 ± 0.005
55	>100	>100	1.33 ± 0.06

* Time required for retraction of anterior segments after injection as % of time required for Ringer's-injected control.

** Time required for tanning of whole larvae after injection as % of time required for Ringer's-injected control.

*** Quotient of the pretanning period of the posterior part of ligated larvae over that of the anterior part. The same notations are used in the subsequent tables.

The cytosol fraction of the CNS was injected into intact and ligated hind parts of red-spiracle larvae (Table II). Injection of 10 μg of cytosol protein per host accelerated tanning to such an extent that intact hosts were still crawling when tanning had already set in. The effect of this quantity of cytosol on retraction was relatively small. Thus, it appears that CNS extract has a greater effect on tanning than on retraction of the anterior segments whereas the opposite is true of hemolymph.

Precipitation of active factors with ammonium sulfate

The hemolymph proteins precipitated with various concentrations of ammonium sulfate were assayed for ARF and PTF activities. The 45–70% ammonium

TABLE II

Effect of injection of various quantities of cytosol fraction of CNS homogenate into red-spiracle larvae of S. bullata

Quantity of cytosol protein of CNS injected (μg)	Retraction % Control	Tanning	
		Control	P/A
0 (Ringer sol.)	100	100	1.28 ± 0.01
10	86 ± 5.0	74 ± 3.6	0.73 ± 0.05
5	95 ± 2.1	79 ± 4.1	0.80 ± 0.0
3	>100	85 ± 1.61	0.91 ± 0.06
2	>100	>100	1.02 ± 0.03
1.5	>100	>100	1.10 ± 0.005
0.75	>100	>100	1.18 ± 0.02

sulfate fraction retained about 75% of the activity of both factors and, most importantly, did not darken and subsequently become toxic to the test animals, so this material was used for further fractionation. Ninety μg of protein from a 45–70% ammonium sulfate fraction had to be injected to get ARF activity equivalent to 1 unit, and 4 times more protein had to be injected to get a similar amount of PTF activity.

Ammonium sulfate precipitation of the cytosol fraction of the CNS was unsuccessful and very little PTF and ARF activities were retained in a 0–70% ammonium sulfate precipitate. Therefore, attempts to precipiate the activity were discontinued, and the original cytosol fraction was used for further fractionations.

Ultrafiltration

The 45–70% ammonium sulfate fraction of hemolymph was filtered through Amicon permselective membranes and tested for activity. The ARF activity passed through an XM-300 filter but was retained by an XM-100 filter indicating that the molecular weight of ARF was between 100,000 and 300,000. The fraction retained by XM-300 showed all of the tanning (PTF) activity.

The cytosol fraction of the CNS was filtered through Amicon filters and the fractions retained by the filters were assayed for activity. The fraction retained by an XM-300 filter (molecular weight above 300,000) retained all the PTF activity, and also showed slight ARF activity when higher concentrations of the fraction were injected.

Taken together the ultrafiltration experiments indicated that the gross molecular weight of ARF of hemolymph is between 100,000 and 300,000; and that of PTF from hemolymph and CNS is above 300,000.

Heat treatment

The 45–75% ammonium sulfate fraction of the hemolymph from orange puparia was dialyzed overnight and diluted 10 times with Ringer solution. Aliquots were then heated for 3 minutes at 60°, 80° and 100° C, then cooled and centrifuged at 10,000 *g* for 10 minutes. At 60° C there was no precipitation but the sample turned milky. At 80° C there was slight precipitation and at 100° C most of the proteins had precipitated. After centrifugation the supernatants were concentrated by filtering through an XM-50 filter and asasyed for ARF and PTF activities. The results indicated that ARF was destroyed by heating at 100° C whereas PTF was not. Heating at 100° C for 60 minutes still gave considerable PTF activity upon injection of 5 μg per larva.

Since the ultrafiltration studies showed that the fraction retained by the XM-300 filter had tanning activity and heating experiments revealed that PTF was not destroyed by heat treatment, the question arose as to whether heating had any effect on the size of the molecules which were originally quite large. As revealed in Table III, a high PTF activity is present in the unheated material as well as in the supernatant of the preparation heated to 100° C. In the unheated material PTF activity was present only in the fraction retained by XM-300 whereas after heating to 100° C almost all the activity was present in the fraction which passed through XM-100 but was retained by the XM-50 ultrafilter.

TABLE III

Comparison of the effect of injection of the active ammonium sulfate (45–70%) fraction of hemolymph before and after heat-treatment at 100° C for 3 minutes. The fractions were filtered through Amicon ultrafilters before assaying for tanning activity. The figures in parentheses are quantities of protein (μg) injected per host. The figures are means from 20 observations

Molecular weight of the fraction	P/A*	
	Unheated, 45–70% fraction	Supernatant of heated 45–70% fraction
Unfractionated	0.86 (600)	0.71 (30)
>300,000 daltons	0.88 (60)	1.0 (30)
100,000 to 300,000 daltons	1.00 (60)	1.16 (30)
50,000 to 100,000 daltons	1.21 (60)	0.76 (30)
Less than 50,000 daltons	1.19 (60)	1.22 (30)

* Ringer injected controls had a P/A ratio of 1.26.

In order to see whether the tanning activity is stable to heating, aliquots of the cytosol fraction of the CNS homogenate were heated for 3 minutes at 60° C, 80° C and 100° C. The fractions turned milky but there was no precipitation. The entire milky solution was assayed for PTF activity and the results clearly demonstrated that tanning activity was not lost after heating to 100° C for 3 minutes. There also remained an apparent, albeit very slight acceleration of retraction of the anterior segments after heating to 100° C. This will be taken up in some later experiments (Table IV).

To find out whether heating broke down the cytosol PTF molecules, the heat treated fractions were filtered through XM-300 ultrafilters and their protein content determined. There was no change in the protein content of the fraction retained by XM-300.

Enzyme hydrolysis

Treatment of the 45–70% ammonium sulfate fraction of hemolymph with trypsin and pepsin for 1–2 hours at 37° C revealed that both ARF and PTF are inactivated by these proteolytic enzymes.

Fraenkel *et al.* (1972) reported that PTF activity of CNS was somewhat resistant to pepsin. This was investigated in greater detail with the XM-300

TABLE IV

Effect of injection of heat-treated cytosol fractions of CNS into intact and ligated hind parts of red-spiracle larvae of S. bullata

Injected fractions	Quantity of protein injected (μg)	Retraction % control	Tanning P/A
Ringer solution	0	100	1.3 ± 0.05
Cytosol, unheated	15	76 ± 2.6	0.66 ± 0.02
Cytosol, heated to 60° C for 3 min.	15	74 ± 3.2	0.73 ± 0.02
Cytosol, heated to 80° C for 3 min.	15	91 ± 2.9	0.70 ± 0.09
Cytosol, heated to 100° C for 3 min.	15	93 ± 1.6	0.79 ± 0.00

retained fraction of CNS cytosol, and the previous observation confirmed. However, ultrafiltration of the pepsin treated fraction through XM-300 membranes revealed that digestion was incomplete over the time of the experiment, and that about half of the material with a molecular weight of above 300,000 had not been cleaved. Hence the apparent PTF activity after enzyme activity could be due to the presence of undigested molecules.

These data on the effect of proteolytic enzymes indicate that both ARF and PTF activities are destroyed after enzyme hydrolysis, which is one more evidence of their protein nature.

Gel filtration of hemolymph fraction

Since the results of the ultrafiltration experiments indicated that the approximate molecular weight range of the ARF active material was between 100,000 and 300,000, Bio-Gel P-300 was chosen for gel filtration. The 45–70% ammonium sulfate fraction was redissolved in Ringer solution and about 250 mg protein applied to a column with a bed volume of about 75 ml. One milliliter fractions were collected at a rate of 3 ml per hour.

The protein content of each fraction after the void volume (27 ml) was determined and every third sample was tested for ARF activity by injecting a volume equivalent to 15 μg of protein into each red-spiracle larva. The fractions eluted between 41 ml and 47 ml showed high ARF and tanning activity. These were pooled, concentrated by filtering through an XM-100 filter and then used for electrophoresis. From a plot of molecular weight as a function of elution volume using standard proteins with known molecular weights such as catalase (240,000), lactic dehydrogenase (140,000) and alkaline phosphatase (80,000) (Fig. 1) the molecular weight range of the active fractions was found to be between 180,000 and 220,000.

Electrophoresis

About 500 μg of hemolymph protein from the ARF active fractions after gel filtration was layered on each gel tube and electrophoresed till the marker dye reached about 0.5 cm from the bottom of the gel. The gels were then fixed, stained, and examined. No difference could be seen between whole hemolymph and gel filtration fractions with regard to the number and position of bands. In each case there were about 13 bands (Fig. 2A & B).

To examine PTF activity, the 45–70% ammonium sulfate precipitate was heated to 100° C for 3 minutes, the supernatant concentrated by filtering through an XM-50 ultrafilter and the material remaining above the filter electrophoresed. There were only four major bands in the gel (Fig. 2C).

The cytosol fraction of the CNS and the fraction that was retained by the XM-300 filter were electrophoresed by the same procedure. In both cases the bands were very faint. There were about 11 bands in the whole cytosol fraction and only 5 in the fraction retained by the XM-300 filter (Fig. 2D & E). The pattern was obviously different from that of the hemolymph fraction.

In order to extract the hemolymph proteins from the gel, 7 gels were run simultaneously using the ARF active fractions of gel filtration and at the end of

electrophoresis the gels were removed from the tubes and one of them was fixed and stained in Coomassie brilliant blue. The other six gels were sliced into two unequal lengths, the upper (cathodal side, slice #II of 4 cm in length), and lower (anodal side, slice #I of 3 cm in length), and the slices pooled. Elution and testing of the proteins from these pooled fractions showed that the lower part of the gel, *i.e.*, slice #I, was more active than the upper part (Table V).

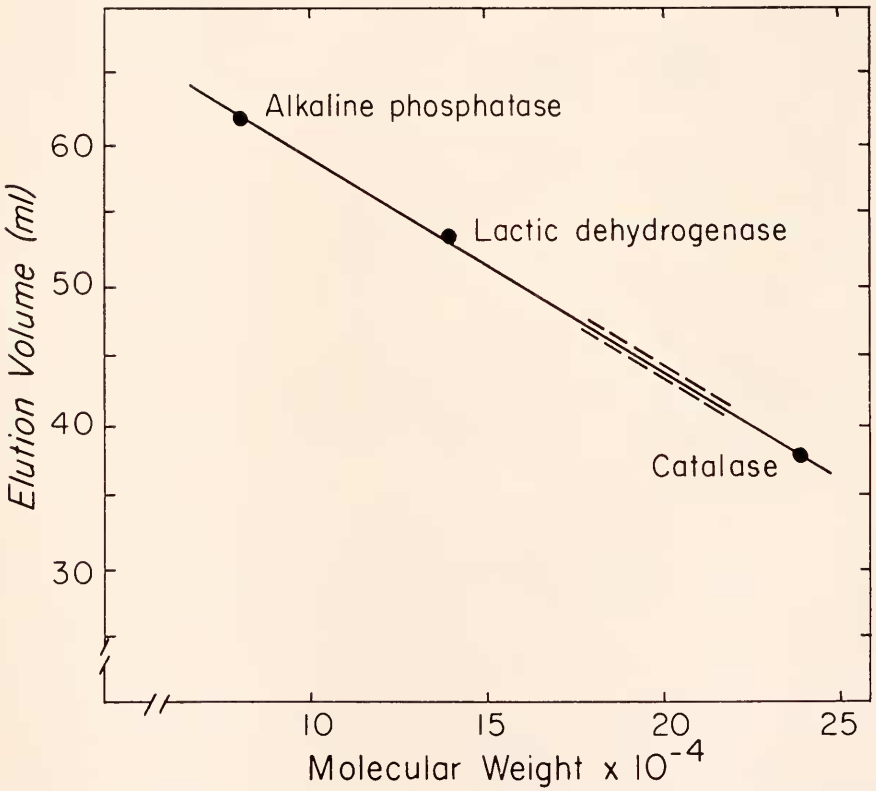

FIGURE 1. Molecular weight determination based on the flow rate through Bio-Gel P-300 column. Total bed volume was 75 ml. Flow rate was 3 ml per hour. The area in dotted line represents the region where the hemolymph fractions were active for ARF.

Knowing that ARF activity was present in the lower part of the gel, other gels were run and sliced into 14 equal parts of 0.5 cm length each (the total length of the gel was usually about 7 cm). Each slice was crushed into smaller bits in 1 ml Ringer solution, extracted overnight at 0–4° C, and the eluants tested as usual. The slices were numbered 1–14 beginning from the anodal end. As shown in Table V a relatively high ARF activity was seen in the extracts of slice #5.

The next step was to electroelute the proteins directly from the gel. Since the approximate location of the activity was known (slice #5, Table V), gels were electrophoresed for various periods of time after the dye front emigrated out of the gel and examined by staining procedures. It was observed that by about

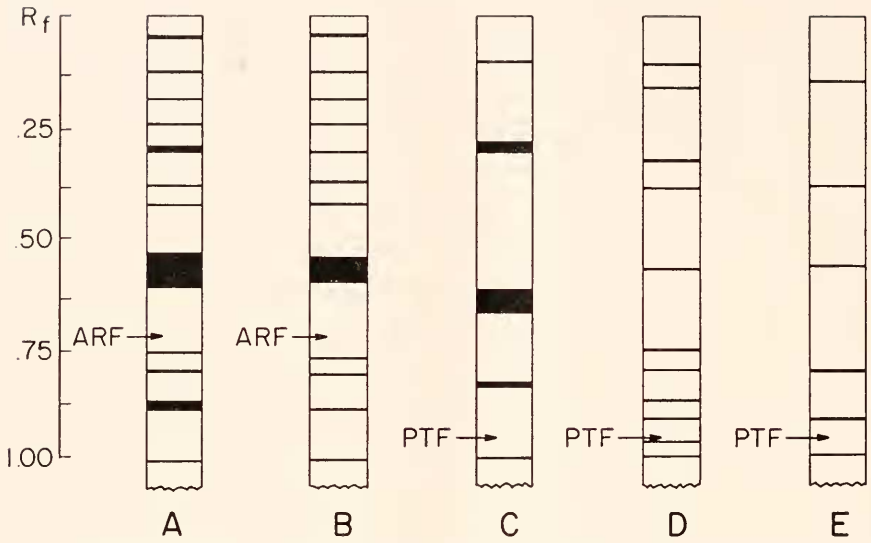

FIGURE 2. Electrophoresis of active fractions from hemolymph and CNS on 7% acrylamide gels. Arrows indicate the active regions; A., Whole hemolymph; B., Bio-gel P-300 active fraction of hemolymph; C., PTF-active supernatant of heat treated ammonium sulfate (45–70%) fractions of hemolymph; D., Cytosol fraction of CNS; E., Cytosol fraction of CNS retained by XM-300 filter.

TABLE V

Effect of injection of extracts obtained from electrophoresis of an active ARF fraction of hemolymph The results have been averaged from 20 observations. The hemolymph was processed in a P-300 column and the active fractions used for gel electrophoresis

Gel slice number*	Amount of protein injected (μg)	% Control time	
		Retraction	Tanning
I (Lower)	10	40.5	66
II (Upper)	10	75.6	82
1	3.5	100	100
2	5	100	100
3	5	100	100
4	13.5	88	91
5	5.5	81	79
6	5.5	95	94
7	12.5	95	97
8	6.5	100	100
9	6.0	96	92
10	3.5	96	94
11	5.5	90	89
12	7.0	100	100
13	9.0	100	100
14	7.0	100	100

* See text.

TABLE VI

Summary of purification steps of anterior segments retraction factor (ARF) from one ml of hemolymph (retraction in 50% of control time equals one Sarcophaga *unit for ARF).*

Fractions	Total protein (mg)	Total number of ARF units	Specific activity (units/mg)	Purification fold	Yield (%)
Hemolymph	205	1990	9.7	1	100
45–70% ammonium sulfate precipitate	125	1388	11.1	1.13	69.7
Bio-Gel P-300 fractions	20	1333	66.6	6.86	67
Electro-eluted fractions	0.56	933	1666	171.7	46.9

3½ hours after the start the active region had migrated to the lowest part of the gel. Therefore, collection of fractions was begun about 3¼ hours after the start of electrophoresis. Ten fractions, of 0.7 ml volume, were collected at intervals of 5 minutes and each was assayed for ARF activity. Fraction #3 collected exactly 3½ hours after the start of electrophoresis seemed to be most active. However, injection of even 6 μg (10 units) of this protein did not accelerate tanning in the ligated hosts, indicating a complete absence of PTF.

A summary of the purification steps of the hemolymph retraction factor is given in Table VI. It has been purified about 170 times. The PTF containing fraction of hemolymph (both the supernatant of 100° C-heated 45–70% ammonium sulfate fraction, and the unheated 45–70% ammonium sulfate fraction retained by the XM-300 filter) were electrophoresed and the gels sliced into 6 equal parts. The proteins were eluted and the concentrated eluants were assayed for PTF activity. In both cases, the eluant from the gel slice closest to the dye front contained the highest PTF activity.

As shown in Table VII, the PTF has been purified 200 times. Since there is no gain in purification by ammonium sulfate precipitation it might be advisable in future to omit this procedure and to prevent darkening of the hemolymph by the same heating step used for purification.

About 300 μg of XM-300 retained protein from the CNS cytosol fraction was electrophoresed in 7% acrylamide gels. The gels were sliced and the proteins eluted, re-concentrated by filtering through XM-300 and, finally, tested for activity. The results showed that the eluant from the slice closest to the dye front contained

TABLE VII

Summary of purification steps of puparium tanning factor (PTF) of 1 ml of hemolymph. A P/A ratio of 1 equals 1 Sarcophaga *unit for PTF*

Fraction	Total protein (mg)	Total number of PTF units	Specific activity	Purification fold	Yield (%)
Hemolymph	205	685	3.3	1	100
45–70% ammonium sulfate precipitate	125	348	2.8	—	51
Supernatant of 100° C heat treated ammonium sulfate precipitate	1.65	330	200	61	48
Eluted from electrophoresed gel slice	0.3	200	667	202	29.2

the PTF activity and injection of 0.6 μg of this protein gave a P/A ratio of 1. The entire series of steps, beginning from the cytosol fraction of the CNS, increased the purity of PTF 5 fold (Table VIII).

The electrophoretic runs of the active fractions from hemolymph and the CNS have shown that ARF and PTF are distinct entities which have different mobilities in gels run under conditions specified above. ARF is located in the middle third of the gel, and PTF in the lowest part of the gel.

Estimation of molecular weight of the active fractions

The molecular weight of the most active electroeluted ARF fraction of hemolymph was determined by SDS-gel electrophoresis. Five separate runs of the sample gave a single band with an average mobility of 0.37. From the semi-log plot of marker proteins and their mobilities (Fig. 3) the molecular weight of ARF was estimated to be about 90,000. Bio-gel P-300 column chromatography showed

TABLE VIII

Summary of purification steps of puparium tanning factor from 100 CNS of mature larvae of Sarcophaga bullata *P*

Fraction	Total protein (mg)	Total number of PTF units	Specific activity	Purification fold	Yield (%)
Cytosol	1.71	570	333	1	100
Cytosol retained by XM-300	0.690	460	667	2	80.6
Eluted from the gel slice	0.120	200	1665	5	35

the molecular weight of the active sample to be between 180,000 and 220,000 (Fig. 1). Since the subunit molecular weight appears to be about 90,000, the molecular weight of the native protein with high ARF activity is probably about 180,000 with 2 subunits of 90,000 each.

By the same procedure, the mobility of the gel-slice eluant of hemolymph having high PTF activity was found to be 0.74, which means a molecular weight of the subunit of PTF of about 26,000. Since the native protein had a molecular weight of more than 300,000, the molecule must have at least 12 subunits with a molecular weight of 26,000 each. (Fig. 3).

The active PTF fraction of the CNS eluted from the gel slice gave a single band with an average mobility of 0.41. From a semi-log plot of the marker proteins and their mobilities (Fig. 3) the molecular weight of PTF was estimated to be about 80,000. Since the native protein had a molecular weight of more than 300,000, this PTF fraction contains at least 4 subunits with a molecular weight of 80,000 each.

Molecular weight determinations by SDS-gel electrophoresis gave different mobilities (Fig. 3) and subunit molecular weights for ARF and PTF. The subunit molecular weight of ARF of hemolymph is about 90,000. The PTF from the CNS has a subunit molecular weight of about 80,000 and that from the hemolymph about 26,000.

Is there ARF activity in the CNS?

The ease of demonstrating PTF activity in the CNS is accentuated by the difficulty of showing ARF activity. In fact, only very high concentrations of the CNS fractions (cytosol and the fraction retained by XM-300 filter) injected into intact

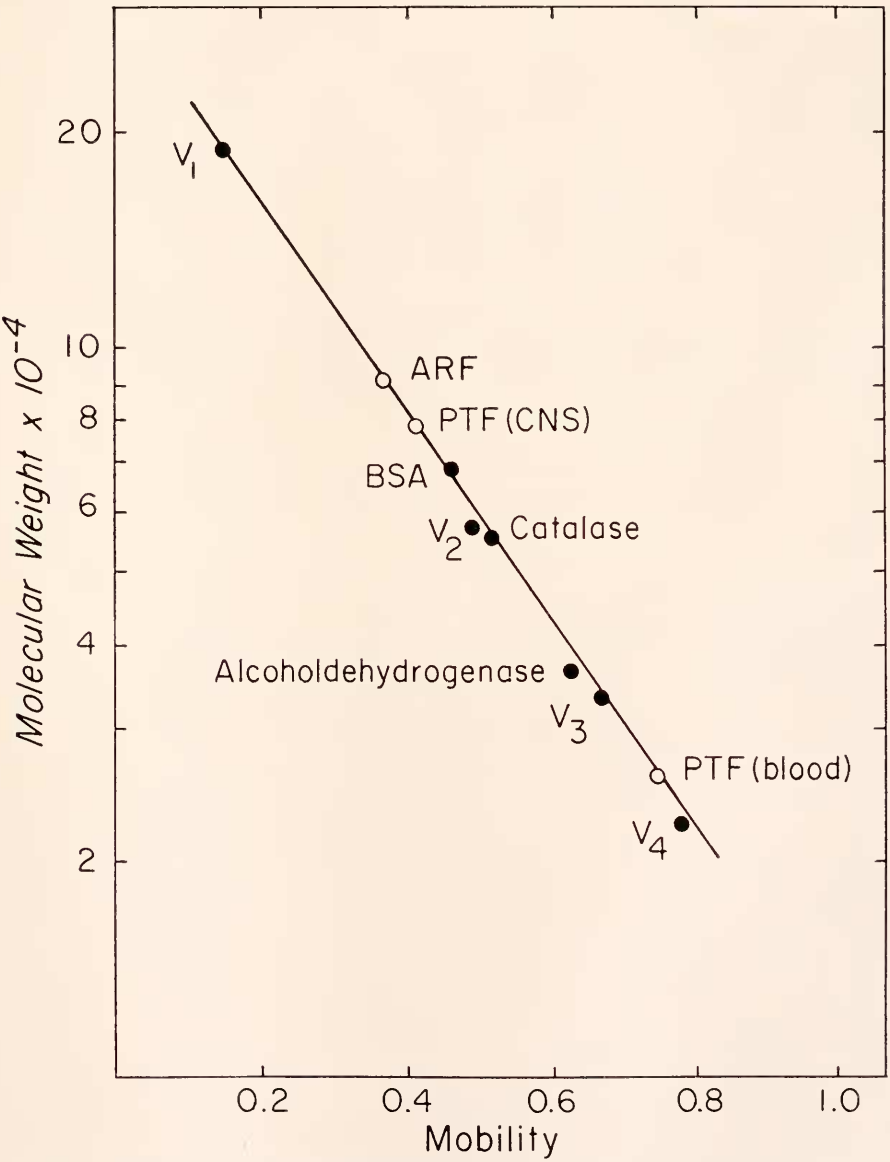

FIGURE 3. Semilog plot of molecular weight as a function of mobility of marker proteins using SDS-gel electrophoresis; V_1–V_4: Proteins of potato yellow dwarf virus with known molecular weights; ARF: Anterior retraction factor; PTF: Puparium tanning factor; BSA Bovine serum albumin.

red-spiracle larvae result in slightly accerelated anterior segment retraction (Table II). There are two possible explanations for this. Either the fractions do contain ARF in low concentration or, alternatively, the highly accelerated tanning and hardening of the cuticle might physically somehow make the hosts withdraw their mouth hooks before controls do. In order to clarify this point the above two fractions were injected with or without α-MDH into intact red-spiracle larvae of *Sarcophaga bullata*. α-MDH ((DL)-α-Methyl-α-hydrazino-β-(3,4-dihydroxyphenyl) propionic acid monohydrate) is a potent DOPA decarboxylase inhibitor and injection of 1 mμ-mole of this material into adult fleshflies has been shown to inhibit tanning almost completely (Seligman, Friedman and Fraenkel, 1969). As shown in Table IX, the cytosol fraction had ARF activity even when the tanning process was inhibited and this activity was lost after heating at 100° C for 3 minutes. However, the fraction retained by the XM-300 filter caused no acceleration of mouth hook retraction, when injected with α-MDH. Therefore the accelerated

TABLE IX

Effect of injection of the CNS fractions with or without α-MDH into red-spiracle larvae of S. bullata. *The figures are means from 20 observations. The quantities injected per host larva were, CNS fraction: 10 μg; MDH: 1 mμ-mole*

Fraction	% Control	
	Retraction	Tanning
Ringer	100	100
α-MDH	100	*
Cytosol	70	68
Cytosol plus α-MDH	81	*
Cytosol heated to 100°C plus α-MDH	100	*
Cytosol retained by XM-300 filter	83	79
Cytosol retained by XM-300 filter plus α-MDH	100	*

* No tanning takes place in presence of α-MDH.

mouth hook retraction that occurs after injection of this fraction is probably not due to the presence of ARF but is due to some physical process that forces the larvae to withdraw their mouth hooks when the tanning process has been greatly accelerated.

The results of this experiment also indicate that the ARF which is present in the whole cytosol fraction probably has a MW below 300 K, since no activity remains after filtration through an XM-300 filter.

Can the hormonal factors (ARF and PTF) replace ecdysone?

In order to see whether or not the hormonal factors can replace ecdysone in whole or in part with regard to pupariation, several active fractions were injected into larvae in 3 different stages either with or without simultaneous injection of β-ecdyosne. Since 0.018 μg of β-ecdysone per larva was reported to be the critical dose for *Sarcophaga peregrina* (Ohtaki, Milkman and Williams, 1967), a subthreshold dose of 0.01 μg and a suprathreshold dose of 0.3 μg of β-ecdysone were injected. The subthreshold dose was used on the assumption that the hormonal

factors might need some ecdysone in order to enable them to penetrate the target tissues.

The fractions were injected at two different periods: either simultaneously with ecdysone or an hour after ecdysone injection. The purpose of injecting an hour after ecdysone was to give the fractions an opportunity to act if they, like ecdysone, have a short half life, but can only function after ecdysone has exerted its action.

The samples injected included (a) 8 μl of active hemolymph from orange puparia (which is equivalent to 15 ARF units and 5 PTF units), (b) five μg each (equivalent to about 10 units each) of ARF and PTF from the fractions eluted from electrophoresed gels, or (c) ten μg (equivalent to 5 PTF units) of cytosol fraction of the CNS.

Three different stages of host larvae used for injections were (i) pre-critical stage larvae, about 36 hours before pupariation which contain no or very little ecdysone, (ii) early post-critical stage larvae, about 12 hours before pupariation which contain some ecdysone and (iii) X-irradiated (10,000 R) pre-critical stage larvae. In the X-irradiated larvae, the synthesis of β-ecdysone is temporarily blocked (Sivasubramanian, Ducoff and Fraenkel, 1974) and hence such larvae are suitable to test the activity of the hormonal factors in the absence of ecdysone.

The results of this experiment were completely negative. Neither the hormonal factors from the hemolymph nor the CNS induced puparium formation. They accelerated puparium formation, as seen in all the previous experiments, only in a red-spiracle stage larva, *i.e.*, long after ecdysone has triggered the initial events of pupariation.

Discussion

In a previous report two alternative models for the hormonal events that occur during puparium formation were proposed (Fraenkel *et al.*, 1972). One of these assumed that the synthesis of "X-factors" which were responsible for the acceleration of puparium formation and tanning was induced by ecdysone, and that the active substances in the CNS were entirely different from those of the hemolymph and merely accelerated the release of the X-factors into the hemolymph. The other model suggested that the X-factors in the hemolymph and CNS were essentially identical substances that were produced in the brain, stored in the peripheral nerve terminals and released into the hemolymph under the influence of ecdysone. The experimental evidence obtained in this investigation support the latter scheme. Based on the results of the present work and the available information in the literature a comprehensive model for the control of puparium formation in fleshflies is presented in Figure 4.

According to this model, the retraction of the anterior segments, the longitudinal contraction to form a barrel shape, and the shrinkage of the cuticle that results in a smooth cuticle are under direct neural control of the CNS. The retraction of the anterior segments is also controlled indirectly through a neurosecretion from the CNS, as is also tanning. The neurosecretory material synthesized in the brain contains 2 distinct entities, the anterior retraction factor (ARF) which controls the retraction of the anterior segments and the puparium tanning factor (PTF) which controls tanning, and these neurosecretions are assumed to be stored in the peripheral nerve endings in all segments of the larva. The molting hormone, ecdysone, whose synthesis and liberation is controlled by another neurosecretion, ecdysio-

tropin, triggers the release of the neurosecretory materials from the peripheral nerves into the hemolymph. Ecdysone also induces the synthesis of various materials such as nucleic acids, enzyme proteins, tanning substrates and other cofactors which enable the released neurosecretory material to bring about retraction of the anterior segments and tanning. Thus, the CNS, both directly and indirectly controls the entire process of puparium formation in flies. Arguments in favor of this scheme will be presented in this discussion.

Of the various processes shown in the scheme, the prothoracotropic effects of neurosecretion and ecdysone-induced protein synthesis during pupariation are well documented in the literature. The involvement of both the CNS and what we now know as ecdysone during molting, and the relationships between the CNS and ecdysone were demonstrated in the pioneering work of Williams (1947). Reports on ecdysone-induced protein synthesis during pupariation, which is a special molting process, abound in the literature (Sekeris, 1965; Karlson and Sekeris, 1966; Sekeri, Sekeris and Karlson, 1968; Arking and Shaaya, 1969; Sahota and Mansing, 1970; Wyatt and Wyatt, 1971; Thomasson and Mitchell, 1972). However, except for the enzyme DOPA decraboxylase which is involved in tanning, the relationship of these proteins to the various morphological events that occur during pupariation is not clear. As will be explained later, at least some of these proteins might coordinate with ARF and PTF in bringing about the retraction of the anterior segments and tanning.

Though no experiments have so far been performed by us, it is clear from the studies of Osborne and his colleagues (Osborne, 1967; Finlayson and Osborne, 1968; Osborne, Finlayson and Rice, 1971) that neurosecretion is stored in nerve endings. Recently they have demonstrated (Osborne *et al.*, 1971) the existence of neurosecretory nerve endings in 3 insects, including the blowfly larva. They suggest that the site of release of neurosecretory material is covered only by a flimsy layer of connective tissue and that the material is probably released into the immediate vicinity of the muscle and diffuses into the hemolymph. They were not sure about the function of the neurosecretory material and assumed that it might have some trophic function.

What causes the release of neurosecretory material from the neurosecretory nerve endings and when it is released is little understood. Ecdysone has been shown to be capable of inducing the release of neurosecretory material from cultured brains of a cockroach (Marks, Itycheriah and Leloup, 1972). Besides, there have been reports on the action of ecdysone on the plasma membrane (Kambysellis and Williams, 1971b). In a previous report (Fraenkel *et al.*, 1972) it was demonstrated that the hormonal factors responsible for the acceleration of the retraction of the anterior segments and tanning can appear in the blood even in the absence of CNS. In that experiment larvae were ligated pre-critically (*i.e.*, no ecdysone was present in the system), and the blood in the ligated hind parts became active after ecdysone injection when the cuticle started to tan. Several experiments in the present investigation have indicated that the hormonal factors in the neurosecretory material of the CNS are essentially identical to those that appear in the hemolymph during pupariation (see below). This, coupled with the fact that the CNS from even much younger larvae (3 day old) contain accelerating factors (Zdarek and Fraenkel, 1969; Fraenkel *et al.*, 1972) tempts us to suggest that the

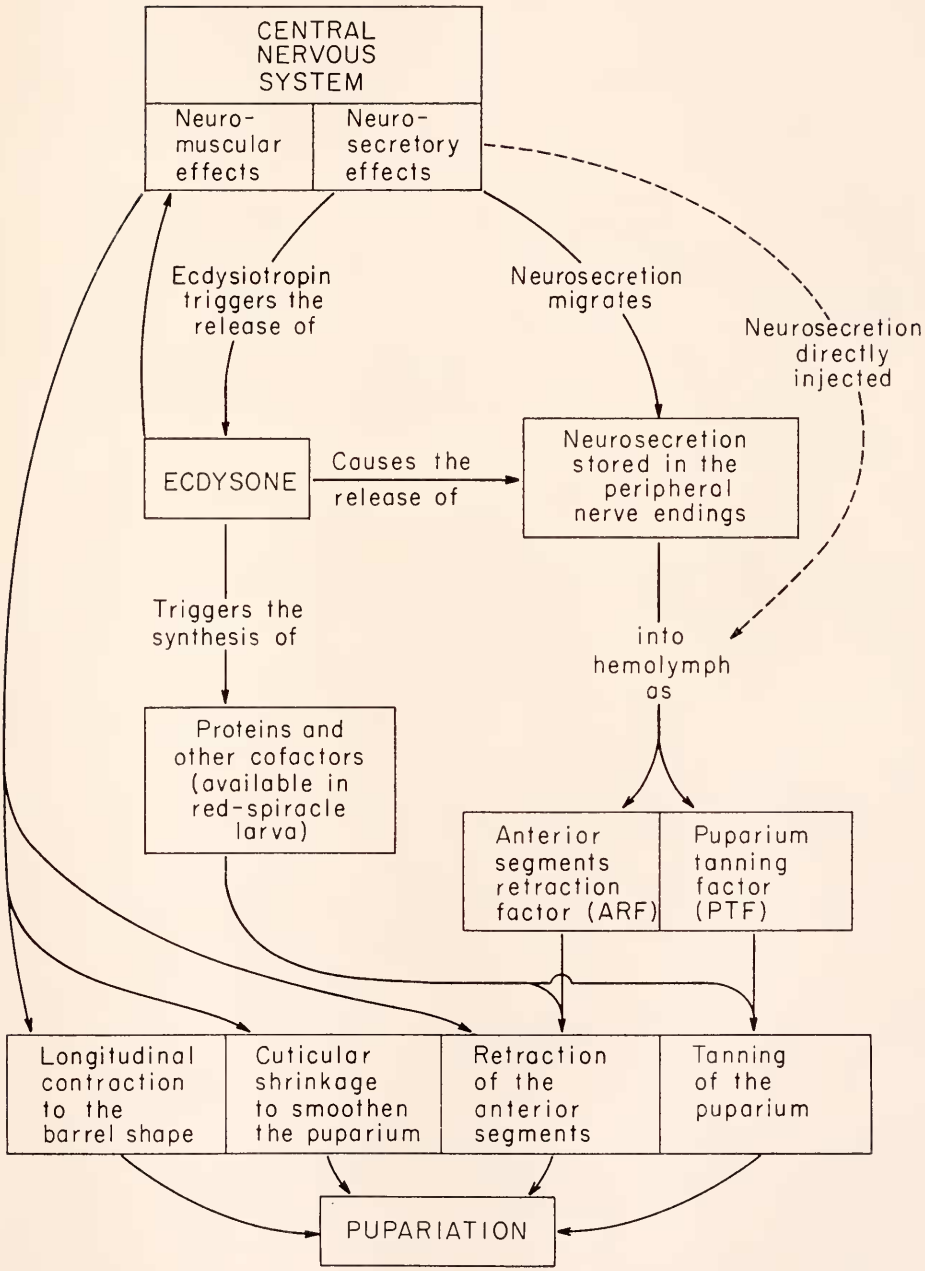

FIGURE 4. A comprehensive scheme of the interrelationships between neuromuscular and neuroendocrine events that occur during puparium formation in the fleshfly *Sarcophaga bullata* P.; explanation in text.

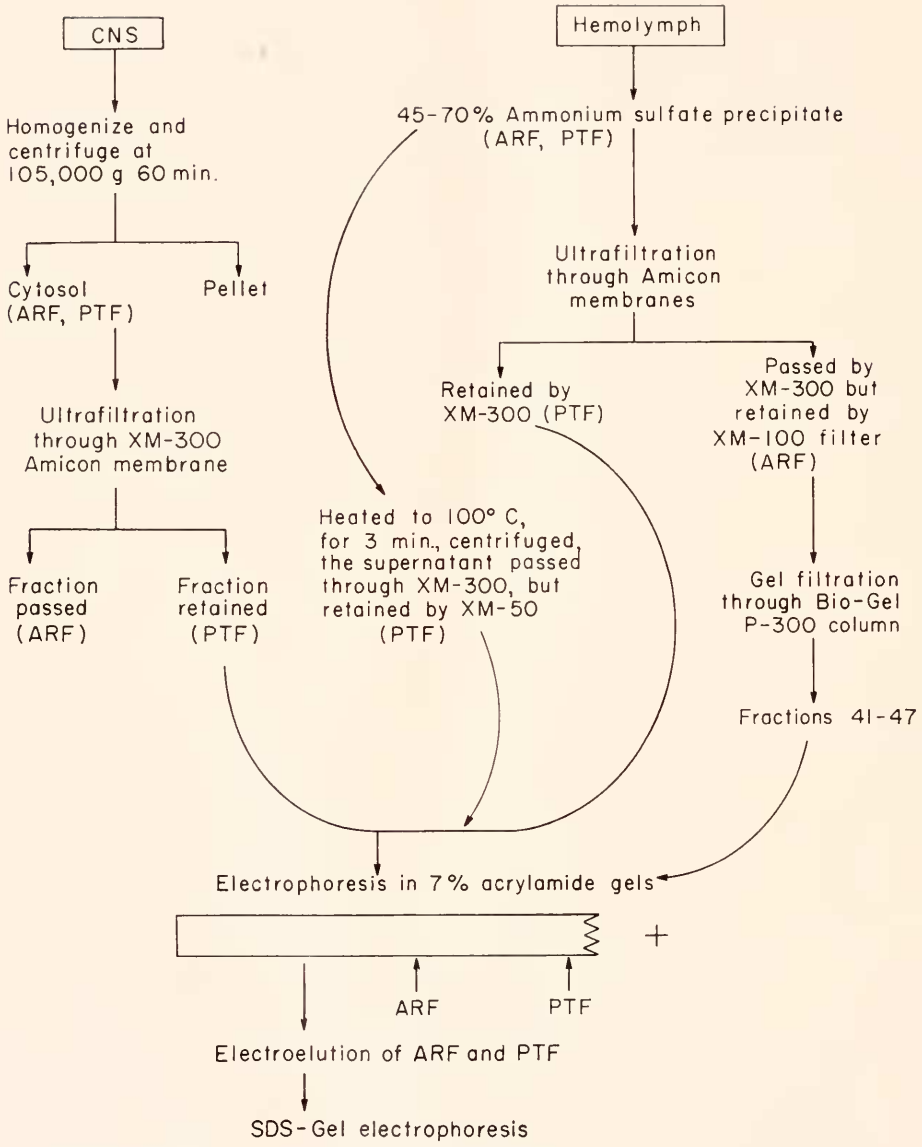

FIGURE 5. Scheme of the steps taken in the isolation of ARF and PTF from the hemolymph and CNS of *Sarcophaga bullata*.

neurosecretory material containing these factors (ARF and PTF) is already stored in peripheral nerve endings in a pre-critical stage larva and is eventually released into the blood by the action of ecdysone prior to puparium formation. This assumption would be strengthened by a demonstration of the disappearance of neurosecretory material from the nerve terminals just prior to pupariation.

TABLE X

Comparison of the effects of various treatments on the retraction factor and tanning factor from the hemolymph of orange puparia of S. bullata

Treatment	ARF	PTF
(1) Heating at 100° C for 3 minutes	Activity lost	Activity remains
(2) Ultrafiltration	Activity passed through XM-300, but retained by XM-100 filter	Activity retained by XM-300 filter
(3) Acrylamide gel electrophoresis	Activity in the middle $\frac{1}{3}$ of the gel	Activity in lower $\frac{1}{3}$ of the gel
(4) Subunit molecular weight as determined by SDS-gel electrophoresis	90,000	26,000
(5) Injection of the purest fraction obtained by electroelution	Accelerates the retraction of anterior segments only	Accelerates tanning only

Direct and conclusive evidence has been obtained to show that there are two distinct factors for retraction and tanning. Figure 5 summarizes the various steps by which the separation of ARF and PTF from hemolymph, and PTF from the CNS was achieved. The properties of the two factors isolated from the hemolymph are different with regard to heating, ultrafiltration, electrophoresis, and subunit molecular weight (Table X).

The CNS extracts, when injected directly into the larva effect the retraction of anterior segments and tanning (Table II), the tanning effect being more predominant than the retraction effect. Nevertheless, the question arises as to whether the factors are identical to those in the hemolymph, and if so, why the tanning effect is predominant. Some of the characteristics which lend credence to the idea that the factors in the two tissues are identical have been listed in Table XI. How-

TABLE XI

Comparison of the properties of the ARF and PTF from hemolymph and CNS extracts

Property	Hemolymph		CNS	
	ARF	PTF	ARF	PTF
Ultrafiltration through XM-300 filter	Passed through filter	Retained by filter	Passed through filter	Retained by the filter
Heating at 100° C for 3 minutes	Destroyed	Not destroyed	Destroyed	Not destroyed
Acrylamide gel electrophoresis	Activity in middle $\frac{1}{3}$ of the gel	Activity in the lower $\frac{1}{3}$ of the gel	*	Activity in the lower $\frac{1}{3}$ of the gel

* Since the concentration of ARF was very low in the CNS extracts it was not isolated and hence the information is not available.

ever, there is a significant difference in heat resistance between CNS and hemolymph PTF's. That from the CNS is not broken down upon heating at 100° C whereas PTF from hemolymph is partially split (Table III). We do not know the reason for this difference.

Although the factors in the CNS and the hemolymph appear very similar, the relative quantities of ARF and PTF are quite different. In unfractionated hemolymph, injection of about 100 μg of protein accelerates retraction of the anterior segments in 50% of control time; but to induce any appreciable tanning activity at least 300 μg must be injected (Table I). However, it appears that the amount of CNS protein to affect PTF activity is one fifth of that which affects ARF activity. Hence, in the CNS the concentration of PTF must be higher than that of ARF and in the hemolymph the opposite must be true. We have no explanation as to why such differences in amount exist when they are secreted from one source.

The studies of Zdarek and Fraenkel (1969) demonstrated that the PTF of the CNS originates in the pars intercerebralis of the brain. Since their experiments were performed with ligated larvae, the existence of ARF in the CNS was not known at that time. However, as described above, ARF also is present in the cytosol fraction of the CNS and it is possible that it, too, originates from the pars intercerebralis. Ecdysiotropin and bursicon also originate from the same region. Are these two hormones related to either of our hormonal factors? It appears that with regard to ARF, the answer is an unequivocal no. ARF has a molecular weight of about 180,000 with two subunits of 90,000 each, whereas the molecular weights of ecdysiotropin and bursicon are well below 50,000. Furthermore, ARF is completely destroyed upon heating at 100° C for 3 minutes while the other two hormones of the brain are partially heat resistant.

What then, of the relationship between PTF and the other brain hormones? With a subunit molecular weight of about 26,000, and resistance to high temperature, it is possible that PTF may be related to ecdysiotropin. However, all previous attempts to isolate ecdysiotropin have ended up with multiple factors and the situation is presently too confusing to make any meaningful comparisons.

PTF as isolated from the CNS is most probably not related to bursicon. It is retained above the XM-300 filter even after heating and has a subunit molecular weight of about 80,000 which is about twice the size of bursicon.

However, the situation with regard to hemolymph PTF is slightly confusing. This fraction, which is initially retained by the XM-300 filter, is partially broken down when heated to 100° C and some of the activity then passes through the XM-300 filter and is retained by the XM-50 filter. After further purification by preparative acrylamide gel electrophoresis both heated and unheated PTF containing fractions appear to have a subunit molecular weight of 26,000. It appears that passage through the gel causes certain changes in the structure of the unheated high molecular weight material. One might argue that the hemolymph PTF has a molecular weight of about 52,000 with two subunits of 26,000 daltons, and that the subunits of this molecule are attached to a bigger molecule leading to its retention on the XM-300 filter when unheated. If this is true, then the question arises as to whether it might be related to bursicon which has a molecular weight of about 40,000 (Fraenkel, Hsiao and Seligman, 1966) and is also a tanning hormone secreted by the pars intercerebralis of the brain. The answer is most probably no because, (a) by heating at 100° C for 3–5 minutes 40–90% of bursicon activity

is lost (Fraenkel *et al.,* 1966) but PTF activity is not (Table IV), and (b) in acrylamide gel electrophoresis bursicon appears at an R_f of 0.3–0.4; but PTF comes very close to the dye front.

Aside from the above, the injection of adult hemolymph active for bursicon into red-spiracle larvae did not accelerate tanning, and conversely, injection of PTF active hemolymph from orange puparia did not induce tanning in neck-ligated newly emerged adult flies (Zdarek and Fraenkel, 1969).

As shown in previous experiments, moderately pure samples taken from acrylamide gels are independent in their action. The ARF fraction effects only the retraction of anterior segments, and the PTF fraction effects only tanning. However, these factors are not effective in pre-critical stage larvae which lack ecdysone, in the same stage larvae when injected with subthreshold doses of ecdysone, nor when injected into early post-critical stage larvae (about 10–12 hours before pupariation). They are effective only if injected into red spiracle stage larvae.

Obviously, the active factors cannot replace ecdysone in effecting pupariation, and it is probable that they need ecdysone-dependent substances (*e.g.,* enzyme proteins, other co-factors), which become available only in the red-spiracle stage, but one can only speculate about the role of such substances in their action.

According to Kambysellis and Williams (1971a, 1971b, 1972), an undialyzable "macromolecular factor" from the hemolymph indispensable for the maturation of spermatozoa in the silkworm, *Samia cynthia,* is present in high titer in the hemolymph during and immediately after pupation. It is inactive in cultures of intact testes in the absence of ecdysone, but promotes spermatogenesis of germinal cysts if the cysts are removed from testes and then cultured. This suggested that the function of ecdysone here is to alter the permeability of the testis walls to facilitate the entry of the macromolecular factor. It is possible that during pupariation ecdysone might function in a similar way, aside from releasing the neurosecretory material from the neurosecretory nerve terminals.

To conclude our discussion of the scheme presented in Figure 4, there is now evidence of direct control by the brain of certain muscular events during pupariation, such as longitudinal contraction to the barrel-shape and cuticular shrinkage to smoothen the puparial surface (Sivasubramanian, Ducoff and Fraenkel, 1974).

Thus, the experimental evidence obtained in the course of this study confirms a previous report with regard to the existence of two different proteinaceous factors which separately effect retraction of the anterior segments and tanning during pupariation. It has also now been demonstrated that the factors in the hemolymph and CNS have a number of common properties, which makes it most probable that the source of the material in the blood is the CNS.

Even though the scheme in Figure 4 so far only applies to a single fly species, *Sarcophaga bullata,* we believe it to be valid for all cyclorrhaphous flies. It is also known that PTF occurs in the corpora cardiaca of *Periplaneta,* the brain of a bug, *Pyrrhocoris apterus,* and the brain of adult flies (Zdarek and Fraenkel, 1969), which suggests that it may play some role in insects which tan after molting. How far the model can be applied to other insects is difficult to predict at this point.

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Summary

Injection of hemolymph from orange puparia or of CNS-extracts from larvae into red-spiracle larvae of *Sarcophaga bullata* accelerates the onset of pupariation in relation to retraction of the anterior segments and tanning. By the use of ammonium sulfate precipitation, heat treatment, ultracentrifugation, ultrafiltration, gel filtration, and electrophoresis, two proteinaceous factors were isolated and partially purified. One of these, called ARF (anterior retraction factor), accelerates retraction and has no tanning activity. The other, called PTF (puparium tanning factor), accelerates tanning and has no ARF activity. The ARF has a molecular weight of about 180,000, is heat labile, and was purified about 170 times. The PTF has a molecular weight of about 312,000, is heat stable, and was purified about 200 times. Similar factors were found in hemolymph and CNS-extracts, however, hemolymph was more active in ARF, and CNS in PTF. These factors are most probably not related to two other brain hormones, ecdysiotropin and bursicon, because of differences in molecular weights, electrophoretic mobilities, and distribution. Based on the similarities in the nature of the ARF and PTF in hemolymph and CNS it is suggested that they originate in the CNS, are stored in peripheral nerve endings, and released through the action of ecdysone into the hemolymph during pupariation.

The factors have no effect in the precritical (36 hours before pupariation) and early post-critical (10–12 hours before pupariation) stage larvae. They are functional only in a red-spiracle larva (2–4 hours before pupariation). This suggests that they cannot replace ecdysone in effecting pupariation, and that they require ecdysone-dependent enzyme proteins or other co-factors which become available only in the red-spiracle larva. The role of such substances may be to alter the permeability of target cell walls in order to facilitate the entry of ARF and PTF.

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